Phenotypic analysis of hMSH2 mutations in mouse cells carrying human chromosomes

Marra, Giancarlo ; D'Atri, S ; Yan, H ; Perrera, C ; Cannavo, Elda ; Vogelstein, B ; Jiricny, Josef

Abstract: Conversion of diploidy to haploidy is a method that allows the generation of stable murine/human hybrid cell lines carrying selected human chromosomes in only a single copy. In this setting, it is possible to detect genetic mutations with greater sensitivity and reliability than in diploid cells. Using this method, we were able to identify mutations in the human mismatch repair (MMR) gene hMSH2 in hereditary nonpolyposis colon cancer families, which have escaped detection by the conventional methods. In this report, we show that such hybrid cell lines can also be a valuable tool in the study of the mutated MMR proteins, in particular the variants found in hereditary nonpolyposis colon cancer families that carry missense mutations and where it is unclear whether they predispose to colon cancer. This analysis is made possible by the fact that the human hMSH2 protein is able to complement the MMR defect in the host murine cell line.

[CANCER RESEARCH 61, 7719–7721, November 1, 2001]

Advances in Brief

Phenotypic Analysis of hMSH2 Mutations in Mouse Cells Carrying Human Chromosomes[1]

Giancarlo Marra, Stefania D'Atri, Hai Yan, Claudia Perrera, Elda Cannavo', Bert Vogelstein, and Josef Jiricny[2]

Institute of Medical Radiobiology, University of Zürich and Paul Scherrer Institute, CH-8008 Zürich, Switzerland [G. M., C. P., J. J.]; Istituto Dermopatico dell'Immacolata, 00167 Rome, Italy [S. D., E. C.]; and Howard Hughes Medical Institute, The Oncology Center, Johns Hopkins University, Baltimore, Maryland 21231 [H. Y., B. V.]

Abstract

Conversion of diploidy to haploidy is a method that allows the generation of stable murine/human hybrid cell lines carrying selected human chromosomes in only a single copy. In this setting, it is possible to detect genetic mutations with greater sensitivity and reliability than in diploid cells. Using this method, we were able to identify mutations in the human mismatch repair (MMR) gene *hMSH2* in hereditary nonpolyposis colon cancer families, which have escaped detection by the conventional methods. In this report, we show that such hybrid cell lines can also be a valuable tool in the study of the mutated MMR proteins, in particular the variants found in hereditary nonpolyposis colon cancer families that carry missense mutations and where it is unclear whether they predispose to colon cancer. This analysis is made possible by the fact that the human hMSH2 protein is able to complement the MMR defect in the host murine cell line.

Introduction

Germ-line mutations in *hMSH2* or *hMLH1* MMR[3] genes are associated with the predisposition to colorectal and endometrial cancers in HNPCC families.[4] A second somatic alteration in the wild-type allele of these genes occurs early in colon carcinogenesis, thus inactivating the MMR system. Because this system is devoted to the repair of nucleotide misincorporations and misalignments occurring during DNA replication, MMR-deficient tumors are characterized by a mutator phenotype with a high frequency of microsatellite instability. Conventional procedures identify germ-line mutations in ~70% of HNPCC kindred. In some cases, the failure to detect mutations has been attributed to the interference of the wild-type sequence, which masks the sequence of the mutated allele. To overcome this problem, we have recently introduced conversion of diploidy to haploidy, a procedure in which lymphocytes of HNPCC patients are fused with mouse cells to obtain stable hybrid cell lines carrying the desired maternal or paternal human chromosome (1). This highly sensitive and reliable method enabled us to identify mutations in MMR genes that had been undetectable using standard mutational analysis. We were interested to find out whether these hybrid cell lines could also be used to study the phenotypic changes associated with the germ-line mutations. To this end, we analyzed the phenotypes of hybrids carrying a single copy of the human chromosome 2 mutated in *hMSH2*. This was possible, because the human hMSH2 protein was able to complement the MMR defect of the recipient murine cells, in which both copies of the *Msh2* gene had been inactivated.

Materials and Methods

Conversion of Diploidy to Haploidy. This procedure was performed as described previously (1). Briefly, lymphocytes from HNPCC patients were mixed with a *hprt*-deficient and geneticin-resistant clone of E2 cells, derived from *Msh2*-deficient mouse embryonic fibroblasts. The cells were fused using a BTX ElectroCell Manipulator, and hybrids resistant to both hypoxanthine-aminopterin-thymidine and geneticin were selected and expanded for genotyping. Microsatellite markers linked to the *hMSH2* locus were used to screen for hybrids containing the maternal or the paternal *hMSH2* allele (1). To test hMSH2 mutations biochemically, several hybrids with either the wild-type or the mutated allele from four HNPCC kindred were expanded in DMEM (Life Technologies, Inc.) supplemented with 10% FCS, 2 mM L-glutamine, and 1 × hypoxanthine-aminopterin-thymidine medium.

Western Blots. Cytoplasmic and nuclear extracts for Western blots and MMR assays were prepared as described previously (2, 3). Fifty μg of the extracts were loaded on 7.5% SDS-polyacrylamide gels, and the separated proteins were transferred onto membranes. These were first blocked with 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Tween 20, and 5% nonfat dry milk for 1 h at 37°C and then incubated for 1 h with the following primary mAbs: anti-hMSH2 mAb NA26 (Oncogene Research); anti-hMSH6 mAb 2D4 (4); anti-hMLH1 mAb13271A (PharMingen); and anti-β-tubulin mAb N357 (Amersham). The latter mAb was used as an internal standard for loading. Immunodetection was carried out by using a horseradish peroxidase-linked anti-mouse secondary antibody and ECL detection reagents (Amersham Pharmacia Biotech).

MMR Repair Assay. The efficiency of the cell extracts in repairing DNA mismatches was tested as described previously (5). Briefly, 5 ng of M13mp2 DNA heteroduplex containing a G/T mismatch in the coding sequence of the *lacZ* α complementation gene were used in a repair reaction together with 50 μg of cytoplasmic extract. The DNA heteroduplex was purified, introduced by electroporation into *Escherichia coli* NR9162 (*mutS* strain), and plated on minimal medium in a soft agar layer containing 0.5 ml of a log phase culture of CSH50 (the α-complementation strain), 0.5 mg of isopropyl-β-D-thiogalactopyranoside, and 2 mg of 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside. After 20 h incubation at 37°C, plaques were assigned to one of the following phenotypes: blue, colorless, or mixed. If no repair occurred, a high percentage of mixed plaques containing both blue and colorless progeny was observed. Reduction of mixed plaques and a concomitant increase in single-color plaques were indicative of repair. Repair efficiency (%) was calculated as follows: 100 × [1 − (% mixed plaques in extract-treated sample)/ (% mixed plaques in extract-untreated sample)]. The data obtained with this method were confirmed by testing nuclear extracts with a different *in vitro* MMR assay (Ref. 3; data not shown).

Temozolomide Sensitivity. The effect of temozolomide on the cell hybrids was evaluated by the tetrazolium salt method (6). The cells were suspended (1 or 2 × 10^4 cells/ml, depending on the cell line) in DMEM supplemented with 10% FCS and 2 mM L-glutamine, dispensed in 50-μl aliquots into 96-well plates, and allowed to attach for 18 h at 37°C. Different amounts of temozolomide (50–1400 μM; Schering-Plough) were then added in 50 μl of culture medium, and plates were maintained at 37°C for 6 days. Four replicated wells were used for controls and for each drug concentration. Sensitivity of hybrids to temozolomide was also evaluated in the presence of O^6-BG (Sigma), a

Received 7/20/01; accepted 9/14/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] The research was supported in part by the Schweizerischer Nationalfonds für die Förderung der wissenschaftlicher Forschung and in part by the Italian Ministry of Health.

[2] To whom requests for reprints should be addressed, at Institute of Medical Radiobiology, University of Zürich, August Forel-Strasse 7, CH-8008 Zürich, Switzerland. Phone: 41-1-634 8910; Fax: 41-1-634 8904; E-mail: *jiricny@imr.unizh.ch*.

[3] The abbreviations used are: MMR, mismatch repair; HNPCC, hereditary nonpolyposis colorectal cancer; mAb, monoclonal antibody; O^6-BG, O^6-benzylguanine; MGMT, O^6-methylguanine-DNA methyltransferase.

[4] A database of the International Collaborative Group on HNPCC is at internet address http://www.nfdht.nl.

specific inhibitor of MGMT. To this end, cells were plated as described above and exposed to 20 μM O^6-BG for 2 h before temozolomide treatment. The same inhibitor concentration was maintained in culture during the 6-day treatment. Control groups were exposed to O^6-BG alone. MGMT activity of O^6-BG-treated cells was evaluated according to the method described by Morten and Margison (7). This activity was undetectable 2 h after the addition of the inhibitor and remained abrogated up to the end of the assay (data not shown). At the end of the incubation period, 20 μl of a 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) solution were added, and the cells were incubated at 37°C for an additional 6 h. They were then lysed with a buffer (0.1 ml/well) containing 20% SDS and 50% *N,N*-dimethyl formamide (pH 4.7). After an overnight incubation, the absorbance was read at 595 nm using a 3550-UV microplate reader (Bio-Rad). Cell sensitivity to drug treatment was expressed in terms of IC_{50} (*i.e.,* the drug concentration capable of producing 50% inhibition of cell growth, calculated on the regression line in which absorbance values at 595 nm were plotted against the logarithm of drug concentration).

Results and Discussion

We studied proteins extracted from several hybrid cell lines carrying *hMSH2* alleles found in four HNPCC families (GJ, FM, W, and SG kindred). Hybrids carrying normal *hMSH2* alleles, as verified by microsatellite analysis and sequencing (1), expressed full-length hMSH2 (GJ8.2, GJ6.5, GJ8.1, FM10.5, W 12, W 26, and SG4 in Fig. 1). The *hMSH2* and *hMSH6* genes are closely linked on chromosome 2p, and it was expected that all hybrids carrying the former gene would also carry the latter. In addition, hMSH6 is stabilized by hMSH2 in the mismatch recognition complex hMutSα (hMSH6/hMSH2; Ref. 8). The presence of hMSH6 expression in each of these hybrids was confirmed by Western blot analysis (Fig. 1). In contrast, hMSH2 was absent in GJ 6, FM1.6, W 6, W 27, W 35, SG16, and SG15 hybrids, which carry the mutated *hMSH2* alleles (Fig. 1). This was anticipated for three of these mutations, an out-of-frame deletion of exon 12 (family GJ), an in-frame deletion of exon 5 (family FM), and a 24-bp insertion between codons 215 and 216 (family W), which bring about destabilization of *hMSH2* mRNA or proteins (1, 9). However, the A636P missense mutation in family SG also resulted in destabilization of hMSH2 (SG16 and SG15 in Fig. 1, verified by a lack of expression of the mutated protein in a baculovirus system), whereas reverse transcription showed full-length mRNA expressed at the same level as in the hybrid with the wild-type allele (data not shown). This finding, which suggests that the A636P mutation also predisposes to colon cancer, could be corroborated by the fact that it was identified in three HNPCC kindred but not in 400 alleles from normal individuals (10).[5] The weak band visible at the level of hMSH6 in extracts of hybrids carrying the mutated *hMSH2* alleles (Fig. 1) represents residual murine Msh6 protein in the *msh2*$^{-/-}$ mouse recipient cells, which cross-reacts with the anti-hMSH6 antibody.

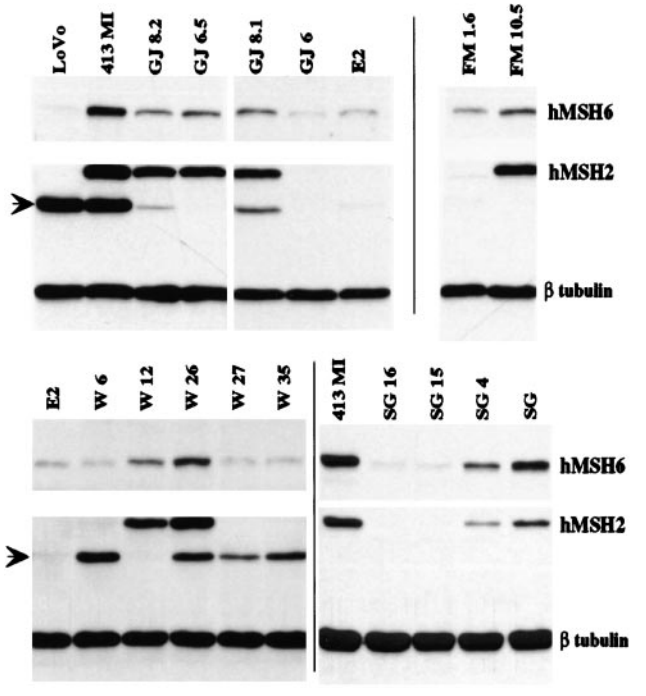

Fig. 1. Western blots showing the expression of hMSH2 and hMSH6 in hybrid cell lines carrying *hMSH2* alleles from families GJ, FM, W, and SG. *LoVo*, human colon cancer line lacking hMSH2 and hMSH6; *413 MI* and *SG*, human lymphoblastoid cell lines immortalized from a normal subject and the index case of the SG family, respectively, were used as a positive control; *E2*, *Msh2*-deficient murine recipient cell line. Hybrids from families GJ, FM, and W were also tested for hMLH1 expression (*arrow*).

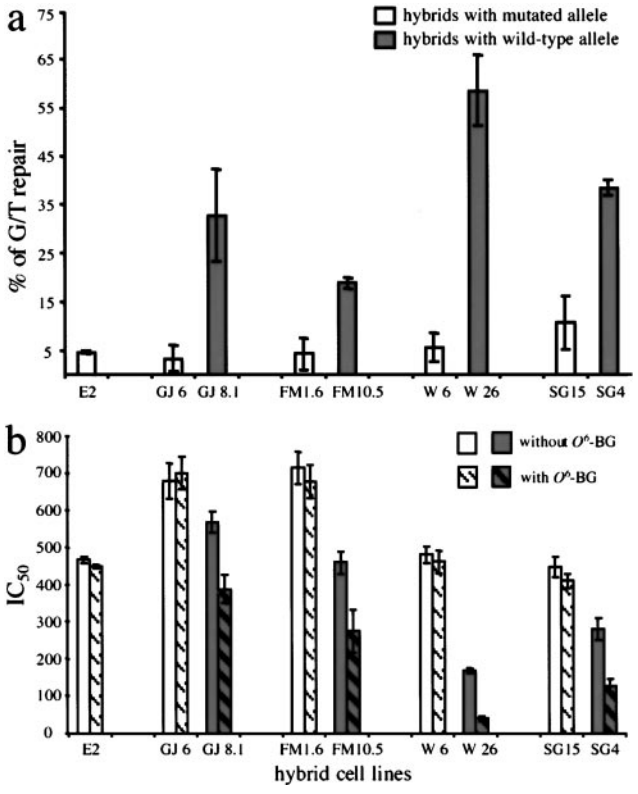

Fig. 2. *In vitro* MMR efficiency and temozolomide sensitivity of hybrids from the four HNPCC families, carrying either the mutated (□) or the wild-type (■) alleles. Each value represents the mean of two (MMR assay) or four (temozolomide sensitivity) independent experiments; *bars*, SE. *a*, efficiency of repair of heteroduplex DNA containing a G/T mismatch. *b*, temozolomide sensitivity of hybrid cell lines evaluated by the MTT assay, either in the absence (*plain columns*) or in the presence (*dashed columns*) of 20 μM O^6-BG, which inhibits the methylation-detoxifying enzyme MGMT and thus augments the difference in sensitivity of each pair of hybrids attributable to the MMR function (see text). Data from the recipient mouse cell line E2 are also shown.

Two hybrids from each HNPCC family, one carrying the mutated allele and the other the wild-type allele, were selected for further analysis. Fig. 2*a* shows that extracts of all hybrid cell lines carrying the wild-type *hMSH2* alleles were proficient in *in vitro* MMR assays. The recipient murine E2 cells do not express Msh2 and are thus MMR deficient. The finding that extracts of hybrids expressing human hMSH2 and hMSH6 are MMR proficient demonstrates that the human hMSH2/hMSH6 heterodimer can functionally interact with the murine MMR factors that are downstream of mismatch recognition. Our finding that the human hMSH2 can interact with its cognate

[5] Internet address: http://www.nfdht.nl.

murine partners emphasizes the high degree of conservation of the mammalian MMR system. It is further supported by the study of Buermeyer *et al.* (11) who were able to complement *Mlh1*-deficient mouse embryonic fibroblasts with *hMLH1* cDNA. The variability in the efficiency of the *in vitro* MMR reactions is also noteworthy. As shown earlier (1), the electrofusion gives rise to hybrids that carry on average 11 ± 3 human chromosomes, and it is possible that some, but not others, express other human proteins that participate in MMR. Indeed, the data in Fig. 1 show that the MMR-proficient hybrids GJ 8.1 and W 26 also contain hMLH1, which is on chromosome 3.

MMR-deficient cells are generally more resistant to killing by DNA-methylating agents than their MMR-proficient counterparts. The primary reason underlying this phenomenon may be linked with futile attempts of the MMR system in the latter cells to repair O^6-methyl-G/T mismatches, which may trigger apoptosis (12). We therefore wanted to test whether the hybrids carrying the mutated *hMSH2* alleles were more resistant to killing by the methylating agent temozolomide than those carrying the wild-type alleles. However, because O^6-methyl-G is efficiently processed by MGMT, which represents the first line of defense against DNA damage caused by methylating agents, the experiments were performed both in the presence and in the absence of O^6-BG, a specific inhibitor of MGMT (Fig. 2*b*). This eliminated the possibility that the observed differences between hybrids were caused by different basal levels of MGMT. When MGMT is inhibited by O^6-BG, MMR-proficient cells become generally more sensitive to killing by methylating agents, whereas MMR-deficient cells tend to retain their basal level of tolerance (12, 13). Fig. 2*b* shows that in the absence of O^6-BG, the hybrid cell lines carrying the mutated *hMSH2* alleles (■) were more resistant ($P < 0.01$, Student's *t* test) to temozolomide than hybrids with wild-type alleles (□). As expected, the tolerance of the MMR-deficient hybrids was not affected by O^6-BG treatment (Fig. 2*b*, ▨), whereas the MMR-proficient hybrids (◪) became more sensitive to temozolomide in the presence of O^6-BG ($P < 0.01$, Student's *t* test).

The functional characterization of inherited MMR gene alterations is often required to distinguish pathogenic mutations from polymorphisms, especially in those HNPCC families where segregation studies are not possible. Such tests have been described (14, 15), but their complexity precludes their use by most clinical laboratories. The fact that human and mouse MMR proteins are complementary (Ref. 11 and this report) allowed us to analyze *in vivo* hybrid cells with a simple laboratory test that reliably distinguishes cells with nonfunctional hMSH2 proteins from cells with wild-type hMSH2. This was possible because the tolerance to methylating agents represents a characteristic phenotype of MMR-deficient cells. Similarly, it should be possible to extend the functional approach proposed in this report to the analysis of hMLH1 mutations by using recipient mouse $Mlh1^{-/-}$ cells and of other genes in genetic diseases where the phenotype of the target protein can be assayed.

Acknowledgments

We thank Tom Kunkel for the gift of the M13 mp2 phage DNA and Patrick Dufner for assistance in the expression of the mutant hMSH2 variants in the baculovirus system.

References

1. Yan, H., Papadopoulos, N., Marra, G., Perrera, C., Jiricny, J., Boland, C. R., Lynch, H. T., Chadwick, R. B., de la Chapelle, A., Berg, K., Eshleman, J. R., Yuan, W., Markowitz, S., Laken, S. J., Lengauer, C., Kinzler, K., and Vogelstein, B. Conversion of diploidy to haploidy. Nature (Lond.), *403:* 723–724, 2000.
2. Marra, G., Chang, C. L., Laghi, L. A., Chauhan, D. P., Young, D., and Boland, C. R. Expression of human MutS homolog 2 (hMSH2) protein in resting and proliferating cells. Oncogene, *13:* 2189–2196, 1996.
3. Holmes, J., Clark, S., and Modrich, P. Strand-specific mismatch correction in nuclear extracts of human and *Drosophila melanogaster* cell lines. Proc. Natl. Acad. Sci. USA, *87:* 5837–5841, 1990.
4. Marra, G., Iaccarino, I., Lettieri, T., Roscilli, G., Delmastro, P., and Jiricny, J. Mismatch repair deficiency associated with overexpression of the *MSH3* gene. Proc. Natl. Acad. Sci. USA, *95:* 8568–8573, 1998.
5. Thomas, D. C., Roberts, J. D., and Kunkel, T. A. Heteroduplex repair in extracts of human HeLa cells. J. Biol. Chem., *266:* 3744–3751, 1991.
6. Hansen, M. B., Nielsen, S. E., and Berg, K. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. J. Immunol. Methods, *119:* 203–210, 1989.
7. Morten, J. E., and Margison, G. P. Increased O^6-alkylguanine alkyltransferase activity in Chinese hamster V79 cells following selection with chloroethylating agents. Carcinogenesis (Lond.), *9:* 45–49, 1988.
8. Iaccarino, I., Marra, G., Palombo, F., and Jiricny, J. hMSH2 and hMSH6 play distinct roles in mismatch binding and contribute differently to the ATPase activity of hMutSα. EMBO J., *17:* 2677–2686, 1998.
9. Liu, B., Parsons, R. E., Hamilton, S. R., Petersen, G. M., Lynch, H. T., Watson, P., Markowitz, S., Willson, J. K., Green, J., de la Chapelle, A., Kinzler, K. W., and Vogelstein, B. *hMSH2* mutations in hereditary nonpolyposis colorectal cancer kindreds. Cancer Res., *54:* 4590–4594, 1994.
10. Yuan, Z. Q., Wong, N., Foulkes, W. D., Alpert, L., Manganaro, F., Andreutti-Zaugg, C., Iggo, R., Anthony, K., Hsieh, E., Redston, M., Pinsky, L., Trifiro, M., Gordon, P. H., and Lasko, D. A missense mutation in both *hMSH2* and *APC* in an Ashkenazi Jewish HNPCC kindred: implications for clinical screening. J. Med. Genet., *36:* 792–793, 1999.
11. Buermeyer, A. B., Wilson-Van Patten, C., Baker, S. M., and Liskay, R. M. The human *MLH1* cDNA complements DNA mismatch repair defects in Mlh1-deficient mouse embryonic fibroblasts. Cancer Res., *59:* 538–541, 1999.
12. Karran, P., and Bignami, M. DNA damage tolerance, mismatch repair and genome instability. Bioessays, *16:* 833–839, 1994.
13. D'Atri, S., Tentori, L., Lacal, P. M., Graziani, G., Pagani, E., Benincasa, E., Zambruno, G., Bonmassar, E., and Jiricny, J. Involvement of the mismatch repair system in temozolomide-induced apoptosis. Mol. Pharmacol., *54:* 334–341, 1998.
14. Shimodaira, H., Filosi, N., Shibata, H., Suzuki, T., Radice, P., Kanamaru, R., Friend, S. H., Kolodner, R. D., and Ishioka, C. Functional analysis of human MLH1 mutations in *Saccharomyces cerevisiae*. Nat. Genet., *19:* 384–389, 1998.
15. Guerrette, S., Acharya, S., and Fishel, R. The interaction of the human MutL homologues in hereditary nonpolyposis colon cancer. J. Biol. Chem., *274:* 6336–6341, 1999.